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(54) Title: METHOD FOR PROTECTING A SUBSTANCE LIABLE TO OXIDATIVE DETERIORATION

(57) Abstract

Antioxidative protection is conferred on a substance liable to oxidative deterioration, by admixing it with at least one compound selected from sub-groups (α), (β) and (γ), namely: (α) rosmarinic acid; (β) salts of the carboxylic acid function in rosmarinic acid; (γ) esters and amides of the carboxylic acid function in rosmarinic acid; subject to certain provisos.

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METHOD FOR PROTECTING A SUBSTANCE LIABLE TO OXIDATIVE DETERIORATION

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to protecting a substance liable to oxidative deterioration and to materials which are so protected.

Artificial antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary-butylhydroxyquinone (TBHQ) and propyl gallate (PG) are known. However, in recent years, there has developed a movement towards avoiding using artificial additives, particularly where foodstuffs and medicaments are concerned. Much effort has therefore been invested in obtaining antioxidants from plants and especially from the *Labiatae* plant family.

Thus, in US 3,950,266 (Chang et al.), there is described a process for extracting antioxidant material from rosemary or sage, using organic solvents (examples of which are hexane, benzene, ethyl ether, chloroform, ethylene dichloride, dioxane and methanol) boiling at up to 100°C, and subsequently carrying out various steps of purification. It appears that in all practical examples, before further purification, the crude antioxidant was washed with water, and then bleached with active carbon. It will be appreciated that antioxidant material produced in this manner will be solvent-soluble and water-insoluble.

By contrast, according to US 4,012,531 (Viani), extraction of plant material is carried out in absence of organic solvents, under mildly alkaline conditions, using a basic aqueous buffer at pH from 7 to about 11.5, preferably in an inert atmosphere. Examples describe the extraction of rosemary, sage and parsley at pH 8.6-9.3, at 55-90°C.

In US 4,450,097 (Nakatani et al.), antioxidant material is isolated from rosemary by extraction with a non-polar solvent, removal of the solvent and steam distillation, giving an aqueous dispersion, which was filtered, antioxidant being obtained from the solid material by further processing including extraction with aqueous alkali at pH at least 10.5. An isolated antioxidant is 7β,11,12-trihydroxy-6,10-(epoxymethano)abieta- 8,11,13-trien-20-one.

US 4,638,095 (Chang et al.) describes the isolation from rosemary of the antioxidant "rosmaridiphenol", which is structurally a dibenzocycloheptene

derivative. This compound was obtained by chromatographic separation (and appeared in the 75:25 diethyl ether/hexane fraction) of a product made by a procedure including solvent extraction and steam distillation.

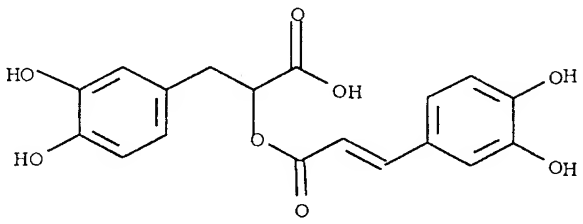
US 4,877,635 (Todd) describes a process for producing an oil-soluble extract of a *Labiatae* herb claimed to contain essentially all of the antioxidant substances in the herb, in the course of which acetone- or methyl ethyl ketone-insolubles, defined as pro-oxidant substances, are, so far as practicable, removed by precipitation. US 5,023,017 (Todd) relates to a stable *Labiatae* antioxidant solution, having a pH 8.4-11.8, prepared from an initial solvent extract of the herb, and claimed to contain (besides <75% water, and an edible alcohol and/or polyol) essentially all of the antioxidant substances in the herb, which is preferably rosemary, sage or thyme.

US 5,433,949 (Kahleyss et al.), after referring to US 5,017,397 which describes preparation of antioxidants by extracting *Labiatae* species with CO_2 at 350-1000 bar and subsequent fractionation, proposes to prepare antioxidants from a similar source by a multistep process utilizing extraction with CO_2 at 80-300 bar, treating the extraction residue with a C_{1-4} alcohol or C_{5-7} hydrocarbon followed by active carbon, and washing out the resultant solvent extract with water to remove color, aromatics and remaining solvent.

It is evident that the various methods known to the prior art for obtaining antioxidants from plants of the *Labiatae* family have for the most part been based on the assumption that the desired materials are to be found in water-insoluble extracts obtained by extracting with non-aqueous solvents, particularly water-immiscible solvents, and in many of the relevant patents, aqueous fractions are rejected. Moreover, in Todd '635, acetone-insolubles are removed, being considered to contain undesired pro-oxidants, and in Todd '017 it is again emphasized that the acetone insoluble materials do not contain antioxidants. Although, as has been noted above, the Viani patent describes direct extraction of the plants with buffered aqueous alkali; the product on evaporation gives a mixture of the desired product with inorganic materials, separation from which entails at least a further step involving crystallization, ion-exchange treatment or acidification. Moreover, the present inventor has found that the product

precipitated on acidification on Viani's alkaline solution is essentially water-insoluble.

In US 4,354,035 (Christ et al.), there is described a process for isolating rosmarinic acid (of formula depicted below)



from essentially water-immiscible organic solvent extracts of an aqueous extract made by extracting *Melissa officinalis* with water at 80-100°C, and acidifying to pH 2-2.5. This patent does not suggest that an industrially useful water-soluble product could be made directly from aqueous extracts of *Melissa officinalis*, without requiring use of water-immiscible organic solvent extracts.

According to the Christ et al. patent, rosmarinic acid is valuable in view of its antiinflammatory properties, see e.g. US 4,329,361 (Zenk et al.). However, rosmarinic acid is also known for its use in skin-treatment or cosmetic compositions, see e.g. JP 63162611, JP 9067251 and US 5,393,526 (Castro). Moreover, rosmarinic acid is claimed to be useful in a composition for protection against erythema and inflammatory reactions caused by exposure to UV rays, and to have antioxidant, antibacterial and antifungal activity, see FR 2652001 (cf JP 06145034). The action of rosmarinic acid as a 5-lipoxygenase inhibitor is also featured in JP 1121217, where, extracted from perilla species, it is used as a constituent of an antiallergic food.

Mention should also be made of the Albeck et al. and Grossman et al. patents, in particular U.S. Patents Nos. 4,857,325, 4,923,697, 4,986,985, 4,997,666 and 5,124,167, which relate to the preparation of antioxidants by aqueous extraction of plant material specifying certain plant families and species, and utilization of such antioxidants. These patents neither disclose nor suggest that antioxidants might be obtained by similarly extracting plants of the *Labiatae* family, nor do they identify the chemical structure of the antioxidants.

The entire contents of the above-mentioned U.S. and other Patents, or published patent applications, are incorporated herein by reference.

Contrary to what is to be expected from the prior art, it has surprisingly been found by the present inventor that useful water-soluble and acetone-insoluble antioxidant material may be obtained from plants of the *Labiatae* family by extraction, which may be carried out at ambient temperatures, with aqueous extractant, by a process in which use of buffered alkali and a step of separation from admixed salts, need not arise. The water-soluble antioxidant material extracted and the extraction process are the subject of our copending patent application 97074.

Although the Viani patent, for example, mentions rosmarinic acid, together with carnosic acid and pro-oxidant flavones, as extracted from rosemary at pH 8.5, there was no inference drawn either in this patent or in FR 2652001 or JP 1121217, or in other prior art, that rosmarinic acid (or its derivatives) could *per se* be viable for antioxidant applications, i.e. to inhibit oxidation of other substances.

Considering also, in general, the vast effort that has in the past been devoted to extraction of plants of the *Labiatae* family, the awareness that useful oil-soluble antioxidants are present in the extracts and the knowledge that rosmarinic acid may also be extracted from these plants, it is surprising that there has been, to the best of Applicants' knowledge, no recognition that this acid or its salts have worthwhile antioxidant activity *per se*, enabling it to be used as an industrial antioxidant and consequently there appear to be no existing patents on this subject.

It is therefore an object of the present invention to provide protection for a substance liable to oxidative deterioration by inclusion in the substance of rosmarinic acid or a derivative thereof, and to provide substances so protected.

It is moreover an object of the present invention to provide protection for a substance liable to oxidative deterioration by inclusion of water-soluble antioxidant material extractable from the plants in question, by using weakly acidic, neutral or alkaline aqueous extractant.

It is a further object of the present invention to provide such protection by inclusion of a material which is highly efficient in terms of antioxidant activity, as compared with many known antioxidants used in industry.

Also, in view of much of the prior art on this subject, the inventor has unexpectedly found that conventionally rejected aqueous residues from the plant

material in question, which has already been solvent extracted to remove water-insoluble solutes (including antioxidants such as carnosic acid), may nevertheless afford useful antioxidant material. Thus, it is yet a further object of the present invention to provide protection for a substance liable to oxidative deterioration by inclusion of water-soluble antioxidant material prepared by a process for extraction of water-soluble antioxidant material from aqueous residues of known extraction processes wherein plants of the *Labiatae* family have already been subjected to extraction to remove water-insoluble solutes.

A still further object of the invention is to provide a process protection for a substance liable to oxidative deterioration by inclusion of water-soluble antioxidant material prepared by permitting maximum recovery of the industrially useful components from plants of the *Labiatae* family, such components including essential oils, completely water-soluble antioxidant material and known components which are both water-insoluble and organic solvent soluble, such as vitamin E and carnosic acid.

Other objects of the invention will become apparent from the description which follows.

SUMMARY OF THE INVENTION

The present invention accordingly provides in one aspect a substance liable to oxidative deterioration, which is characterized by the fact that it incorporates as antioxidant for that substance, at least one compound selected from sub-groups (α), (β) and (γ), namely:

(α) rosmarinic acid;

(β) salts of the carboxylic acid function in rosmarinic acid;

(γ) esters and amides of the carboxylic acid function in rosmarinic acid;

provided that said substance excludes a pharmaceutical formulation, cosmetic composition, animal feedstuff, and a foodstuff and a food composition adapted for human consumption, in any of which said at least one compound was incorporated as a pharmacologically active ingredient in presence or absence of any other pharmacologically active ingredient; and that

said at least one compound excludes mixtures in a form as extracted from natural sources and comprising any compound in sub-groups (α), (β and (γ), together with any other antioxidant(s).

The above proviso, in which "pharmacologically active ingredient" includes such ingredient intended for skin improvement or protection, excludes from the scope of the invention, for example, the disclosures of US 4,329,361, where rosmarinic acid was used in a daily dose of 50-1000 mg for its antiinflammatory properties; use of rosmarinic acid or its salts in skin cosmetics based on its wound treating effect (JP 63162611) or anti-aging effect (US 5,393,526); use of rosmarinic acid in a composition for protection against erythema and inflammatory reactions caused by exposure to UV rays (FR 2652001); and inclusion of rosmarinic acid in an antiallergic food connected with its 5-lipoxygenase inhibiting activity (JP 1121217).

The above proviso further excludes from the scope of the invention use of mixtures of rosmarinic acid or any of its derivatives with any other antioxidant(s) (such as carnosic acid or carnosates) in a form as extracted from natural sources. (see e.g., Viani).

In another aspect, the invention provides use of at least one compound selected from sub-groups (α), (β) and (γ), namely: (α) rosmarinic acid; (β) salts of the carboxylic acid function in rosmarinic acid; (γ) esters and amides of the carboxylic acid function in rosmarinic acid; in admixture with a substance liable to oxidative deterioration, in at least a minimum amount necessary to confer antioxidative protection on said substance, provided that when said substance is a dermato-cosmetic composition, said at least one compound is used in an amount less than 0.1% of the admixture; and that said at least one compound excludes mixtures in a form as extracted from natural sources and comprising any compound in sub-groups (α), (β and (γ), together with any other antioxidant(s).

In yet another aspect, the invention provides a method for treating a substance liable to oxidative deterioration, in order to confer antioxidative protection on said substance, which comprises admixing said substance with at least one compound selected from sub-groups (α), (β) and (γ), namely: (α) rosmarinic acid; (β) salts of the carboxylic acid function in rosmarinic acid; (γ) esters and amides of the carboxylic acid function in rosmarinic acid; in at least a minimum amount

necessary to confer said protection on said substance, provided that when said substance is a dermato-cosmetic composition, said at least one compound is used in an amount less than 0.1% of the admixture; and that said at least one compound excludes mixtures in a form as extracted from natural sources and comprising any compound in sub-groups (α), (β and (γ), together with any other antioxidant(s). The proviso as to the dermato-cosmetic composition excludes the disclosure of FR 2,652,001, in which rosmarinic acid is present in an amount of 0.1-5.0%.

The term "antioxidative protection" in the present specification and claims is intended to include partial as well as substantially total inhibition of oxidation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates chromatographic fractionation of antioxidant material from rosemary, having utility in accordance with an embodiment of the invention.

Figure 2 illustrates chromatographic fractionation of antioxidant material from oregano, having utility in accordance with an embodiment of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The substance incorporating antioxidant according to the invention may be further characterized by at least one of the following features:

(a) it incorporates additionally an emulsifying, dispersing or suspending agent;

(b) it incorporates said antioxidant in a form selected from completely water-soluble materials containing a major proportion of non-antioxidant diluent, and which are solid at ambient temperatures, and aqueous solutions thereof;

(c) it is in the form of a powder, tablet, capsule, solution, emulsion, concentrate, syrup, suspension, gel or dispersion;

(d) said antioxidant has been subjected to chromatographic enrichment or purification, prior to incorporation in said substance;

(e) said antioxidant comprises at least one of:

(i) a sodium rosmarinate, preferably isolated from tissue of plants of the *Labiatae* family, most preferably by use of an aqueous extractant; and

(ii) a rosmarinate salt other than the sodium salt, preferably obtained by cation-exchange with said sodium salt;

(f) said antioxidant constitutes about 0.0001 to about 1.0% by weight of said substance.

It is presently preferred that the substance incorporating antioxidant according to the invention is selected from sub-groups (I), (II) and (III), namely:

(I) pharmaceutical compositions, cosmetic compositions, animal feedstuff compositions and food compositions adapted for human consumption;

(II) an ingredient, liable to oxidative deterioration, adapted to be incorporated into a composition selected from pharmaceutical compositions, cosmetic compositions, animal feedstuff compositions and food compositions adapted for human consumption;

(III) a composition selected from pharmaceutical compositions, cosmetic compositions, animal feedstuff compositions and food compositions adapted for human consumption, which composition comprises an ingredient liable to oxidative deterioration.

Where the substance is a food composition, this may be e.g., selected from sugar-based confectionery, manufactured cereals, fruit or vegetable products, beverages or beverage concentrates, ground meat products and vegetable analogues thereof. Such food composition may comprise at least one additional ingredient selected from the water-soluble vitamins thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid, cobalamin and ascorbic acid; the oil-soluble vitamins retinol, calciferol, tocopherol and menadione, in combined form the elements sodium, potassium, calcium, phosphorus, magnesium, chlorine, sulfur, iron, copper, iodine, manganese, cobalt, zinc molybdenum, fluorine, selenium and chromium, unsaturated fatty acids which are known to metabolized in the body to prostaglandins, and physiologically compatible derivatives of said fatty acids, food colorants and pigments, acceptable dispersing and suspending agents, and water.

When food compositions are preserved with antioxidant(s) according to the invention, an amount of antioxidant(s) effective to prevent oxidation of the ingredient liable to oxidative deterioration, e.g. an oil or fat, or a colorant or pigment, should be used. Generally, from about 0.0001 to about 1%, preferably from about 0.0005 to about 0.1% by weight of the food composition may be used,

depending on the nature of the composition and the type of oxidative activity which is to be inhibited.

Colorants or pigments, used in food compositions, which are liable to oxidative deterioration, are for example, polyene pigments and colorants such as β-carotene.

More especially, food compositions which contain fats or oils comprising fatty acids or their esters, either saturated or unsaturated, may be preserved using antioxidant(s) according to the invention. The fatty acids are well known and are listed in Noller, Textbook of Organic Chemistry, 2nd Ed. pp. 108-113 and 138-146 (1958), which is incorporated by reference herein. Typical but non-limiting examples of food compositions, including fats and oils adapted for use therein, are soybean oil, corn oil, cottonseed oil, olive oil, butter, margarine, salad creams and mayonnaises, dairy products, ice cream, frozen vegetables, soups, fried foods and the like.

A particular aspect of the present invention resides in the use of rosmarinic acid and its derivatives as described herein, for the purpose of antioxidative protection of vitamins and their analogs and functional derivatives of the vitamins and the analogs. In this context there may be mentioned the water-soluble vitamins thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid, cobalamin and ascorbic acid; the oil-soluble vitamins retinol, calciferol, tocopherol and menadione, and their analogs and functional derivatives thereof, especially such analogs and derivatives having analogous biological activity. It is of particular interest, in accordance with an embodiment of this aspect of the invention, that vitamins which are themselves known to be antioxidants, in particular ascorbic acid (including its salts and esters) and α-tocopherol (including its esters) can themselves be protected from deterioration by use of rosmarinic acid and its derivatives.

The substance liable to oxidative deterioration, the oxidation which is inhibited in accordance with the present invention, includes the essential oils, such as those derived, for example, from citrus fruit and flowers

The antioxidants utilized in accordance with the invention are selected from rosmarinic acid, salts of the carboxylic acid function in rosmarinic acid, and esters and amides of the carboxylic acid function in rosmarinic acid. Rosmarinic acid may

be isolated from natural sources e.g. as described in certain of the above-mentioned published documents, or it (and indeed its salts, esters and amides) may be chemically synthesized. It may also be made by enzymatic or cell culture procedures, see e.g. US 5,011,775 (Rao) and published documents mentioned therein, the contents of this patent and of such documents, being incorporated herein by reference.

Without prejudice to the generality of the present invention, it is presently preferred to make rosmarinic acid salts for use in the present invention, as described and claimed in copending application 97074, and as exemplified below.

The invention will be illustrated by the following non-limiting examples.

EXAMPLE 1: Aqueous extraction of raw rosemary plant tissue

(a) A mixture of the dried superficial growing parts of rosemary plants (i.e. stems, leaves and flowers), 2 g, was homogenized with 10 ml water and maintained at ambient temperature (about 24°C) for one hour. The pH of the mixture during this extraction was about 6.5. The supernatant was separated from residual plant tissue and lyophilized to give 0.2 g (10% yield) of a crude completely water-soluble product containing about 10% antioxidant-active substance, the activity of crude product being 430 units/mg.

(b) When the extraction in (a) was repeated, except that citric acid was added initially to give pH 2, it was found that the supernatant had a pH of about 7 and that the crude completely water-soluble lyophilized product had approximately the same antioxidant activity as before.

(c) When the extraction in (a) was repeated, except that NaOH was added initially to give pH 11, it was found that the resultant supernatant had a pH of about 7 and that the crude completely water-soluble lyophilized product had approximately the same antioxidant activity as before.

(d) When the extraction in (a) was repeated, except that the rosemary plant tissue had been treated with steam and extracted with ether, prior to aqueous extraction, it was found that the crude completely water-soluble lyophilized product had approximately the same antioxidant activity as before.

(e) (comparative example) Rosemary plant tissue (5 g) was vigorously stirred with 50 ml Na_2CO_3/$NaHCO_3$ buffer at pH 8.6 and 90°C, essentially as described in

Example 2 of US 4,012,531 (Viani). Extraction of a portion of the reaction mixture with aqueous acetone and lyophilization of the product (after evaporation of the acetone), at this stage, gave a product containing only 5% of the antioxidant material of the present invention (as identified by a sharp peak on HPLC separation and elution with aqueous methanol at between 14 and 15 minutes retention time), thus showing that the desired product remained dissolved in the alkaline medium. A separate portion of the reaction mixture was then acidified as suggested by Viani and as carried out by Todd (see Example 2 of US 5,023,017), both Viani and Todd utilizing only the precipitate as "antioxidant". The precipitate was filtered off and was found to be almost completely water-insoluble. The filtrate, after concentration, extraction with aqueous acetone and lyophilization of the product (after evaporation of the acetone) gave a completely water-soluble antioxidant material, 28% pure in the desired product, according to HPLC. This experiment demonstrates *inter alia* that Viani's aqueous alkaline extract did not contain a completely water-soluble solute.

(f) Rosemary or oregano plant tissue was first subjected to the action of steam at 100°C for about one hour to recover essential oils by steam distillation. Water was added to the residue and the mixture was allowed to stand at 60-100°C for about 2-45 minutes, filtered and the filtrate containing about 4% solute was concentrated to about 40% solute, again filtered and spray-dried. The yellowish powder product contained about 9.5% antioxidant active substance and could be used as such or was subject to chromatographic purification. It was found that while a relatively long work-up time resulted in a darker-colored product (light brown to brown), the high antioxidant activity of the product (about 430 units/mg) was independent of its color. Yield of the powder was as follows:

Run	Extraction conditions	Yield from 25 g plant tissue
1	20 minutes at 80°C	0.7 g (2.8%)
2	45 minutes at 80°C	1.2 g (4.8%)
3	20 minutes at 100°C	1.2 g (4.8%)
4	45 minutes at 100°C	1.4 g (5.6 %)
5	run 4 residue extracted with 125 ml water for 15 minutes at 100°C	+0.4 g (+1.6%)
6	run 5 residue extracted with 125 ml water for 15 minutes at 100°C	+0.28 g (+1.1%)
4-6	(combined)	2.08 g (8.3%)

Example 1 shows *inter alia* that the prior art practice of obtaining antioxidant material from *Labiatae* family plants, from organic solvent soluble extracts, while rejecting aq. fractions, and/or rejecting acetone-insolubles, results in substantial loss of potentially valuable antioxidant. Moreover, Example 1(c) shows surprisingly, that contrary to Viani and Todd, useful completely water-soluble antioxidant material can be obtained by alkaline extraction.

EXAMPLE 2: Method for determination of antioxidant activity

The method is based on the rate of oxidation of linoleic acid (LA) to its conjugated diene hydroperoxide (Pryor et al., in J. Org. Chem., 1993, 58 (13): 3521-3532), and 2,2'-azobis (2-amidinopropane).2HCl (ABAP) is used to provide a constant rate of radical production. A 1% LA aq. emulsion was prepared with 1% Tween 20 and 0.05M sodium phosphate buffer (pH 7.4). A control sample contained 0.025 ml of the LA emulsion + 2.87 ml. of the buffer + 0.1 ml 0.05M ABAP; a test sample contained 0.05 ml of the LA emulsion + 2.7-2.8 ml of the buffer + 0.1 ml 0.05M ABAP + 0.05-0.1 ml of the sample under test. Absorption at 234nm is followed for 5 minutes to establish the uninhibited rate of autoxidation. The test antioxidant in a concentration of 1 mg/ml is then checked to get 50% inhibition, which defines one unit (e.g., if 20 µl inhibits 50%, this is one unit and thus the antioxidant activity is 1000/20 = 50 units/mg). The test antioxidant was added and the inhibited reaction was followed until the antioxidant was consumed

and the rate of the absorption change at 234 nm reverted to that observed at the outset.

EXAMPLE 3: Chromatographic purification and identification of antioxidant

A column of 8cm x 1cm is packed with MacroPrep-methyl (hydrophobic interaction 40 μm, Bio-Rad) was washed exhaustively with water and ethanol, and then used for purification of the water extract lyophilizate (see Example 1(f), above). 200 mg of the lyophilizate was dissolved in 1 ml water, and the solution was loaded on the MacroPrep column that had been pre-equilibrated with water. Elution was carried out using a 0.1% acetic acid/ 70:30 ethanol in water gradient starting with a 100:0 ratio at 0 minutes and ending with a 0:100 ratio at 220 minutes, and at a flow rate of 2.0 ml/minute. Peaks are monitored by UV absorption at 280nm. The column effluent was collected after 85 minutes (193 ml) and the active material was found to be eluted between 85 to 100 minutes (36 ml, 30-40% ethanol (balance water)).

The progressive increase in purity of the above operations was determined by HPLC on RP-18, using a water/methanol gradient starting with a 100:0 ratio at 0 minutes and ending with a 30:70 ratio at 15 minutes, and at a flow rate of 1 ml/minute. Results are shown, together with other relevant purification data, in Figs. 1 and 2 (where "RA" denotes rosmarinic acid, sodium salt) and in the following table, where "% purity" means % in the product in question, of antioxidant material identified by a sharp peak on HPLC separation and elution with aqueous methanol at between 10.68-10.72 mins. retention time:

Fraction	Amount crude antioxidant (mg)	% Purity	Rosmarinate (mg)	Yield (%)
rosemary extract (Fig. 1)	200	10	20	100
21-23	4	81	3.2	16
24-30	15	98	14.7	7.35
31-32	2	86	1.6	8.4
				total = 97.7
oregano extract (Fig. 2)	200	10	20	100
18-22	18	87	15	75
23-27	15	33	4.6	23
				total = 98.0

Both ^{1}H and ^{13}C NMR spectra were measured for the chromatographically pure antioxidant of the invention. It was found that there were only very small shift differences in the chemical shift values for a d_6 -DMSO solution, compared with corresponding data reported for rosmarinic acid (d_6 -acetone for ^{1}H and D_2O for ^{13}C), see Kelley, C.J. et al., J. Org. Chem. 40: 1804 (1975) and *ibid.*, 41: 449 (1976). These differences, taken together with the fact rosmarinic acid is acetone-soluble and the present compound is virtually acetone-insoluble, are consistent with identification of the present product as a carboxylate salt of rosmarinic acid. The compound was identified as sodium rosmarinate, and the formula weight (282) for $C_{18}H_{15}NaO_8$ was confirmed by mass spectra.

EXAMPLE 4: Stability and comparative efficiency of antioxidants

(a) Stability. The lyophilizate obtained from the aqueous extract has a shelf-life with substantially unchanged antioxidant activity of more than two years at 24°C. This lyophilizate (6 mg) was dissolved in 0.5 g glycerol, and 0.25 g of the solution was placed in a tube which was then incubated at 180°C for 15 minutes. A second similar tube was used as a control at room temperature. Following incubation, 1.5 ml water was added to each tube and the activity was checked. There was found

to be no change in the activity in the control and in the tube incubated at 180°C. Moreover, an aqueous extract of rosemary at pH 4 could be kept at ambient temperature for at least 12 months, with retention of antioxidant activity.

(b) Comparative efficiency. Ex. 2 is followed, but it is carried out until antioxidant is consumed and the rate of change of absorption at 234 nm had reverted to that observed at the control. Efficiency is calculated as the time 1 mg of antioxidant continues to inhibit oxidation of linoleic acid. Results are shown in the following table.

Antioxidant	Efficiency of 10 μg (hrs)	Solubility	Toxicity
(prior art:)			
Vitamin E	444	lipid	no
Vitamin C	93	water	no
BHT	814	lipid	yes
Trolox*	650	water	no
PG	500	lipid	yes
TBHQ	432	lipid	yes
Rosemary oil	201	lipid	no
present invention			
98% pure	1413	water	no

*water-solubilized vitamin E

It may be concluded from the foregoing results that the antioxidant of the invention is considerably more efficient than the known antioxidants against which it has been tested. This is true even of the lyophilizate obtained from the aqueous extract as described above and the efficiency of the product is seen to rise dramatically on further purification.

EXAMPLE 5: Antioxidant Effect in Emulsions containing Soybean Oil

(a) Oil in water emulsions were prepared from soybean oil (10%), Tween 80 (7%), Span 80 (3%), water (80%) and antioxidant (0.1 or 0.02%). Antioxidant performance at 50°C as measured by the TBA (thiobarbituric acid) test were as follows:

Antioxidant	Concentration (%)	Days until oxidation
BHA	0.02	2
Vitamin E	0.1	1
Vitamin C	0.1	1
Rosemary Extract*	0.02	4
Rosemary Extract*	0.1	4

*see below

(b) Water in oil emulsions from soybean oil (85%), PGPR emulsifier (Croda, 5%), water (10%) and antioxidant (0.1 or 0.02%) gave test results at 100°C, as measured by the Rancimat (AOM) test, as follows:

Antioxidant	Concentration (%)	Hours until oxidation
BHA	0.02	9.54
Vitamin E	0.1	9.58
Vitamin C	0.1	25.20
Rosemary Extract*	0.02	19.05
Rosemary Extract*	0.1	29.00

*water-soluble, containing 10% sodium rosmarinate; the concentration of the latter in the emulsions is thus, respectively, 0.002 and 0.01.

EXAMPLE 6: Antioxidant Effect in Bulk Oil

A concentrate was first prepared containing 40% lecithin, 40% oil (e.g. soybean oil), 20% propylene glycol and 4% based on the foregoing mixture of 65% (≡2.6% pure) sodium rosmarinate. Rosmarinic acid can alternatively be substituted for the sodium salt.

The thus-prepared concentrate can then be used in a bulk oil which it is desired to protect against oxidative deterioration. Where the oil is soybean oil, test results at 60°C were as follows:

Antioxidant	Concentration	Days until oxidation
(prior art:)		
None		4
Vitamin E	0.06%	4
BHA	0.02%	4
Rosemary oil	0.4%	6
present invention		
sodium rosmarinate	0.005%¤	7
sodium rosmarinate	0.013%♦	8

¤ as 0.2% of the above concentrate
♦ as 0.5% of the above concentrate

EXAMPLE 7: Antioxidant Effect in Emulsions containing β-Carotene

β-Carotene (6 mg), linoleic acid (1 ml) and Tween 40 (2 ml) were dissolved in chloroform, the mixture was concentrated in a rotary evaporator, and the last traces of chloroform were removed by nitrogen. A model emulsion was then prepared by adding double-distilled water (25 ml) to the residue and diluting to 500 ml with phosphate buffer (pH 7.0). A solution (2 ml) of the inventive antioxidant was mixed with 50 ml aliquots of the emulsion, such that antioxidant concentration was 0.005, 0.01 or 0.02%. Comparison samples contained BHA in concentrations of 0.01 and 0.02%, and ascorbic acid (0.1%). A control sample contained no antioxidant but instead, 95% ethanol (2 ml). It was found that over a 100 hour time period, the inventive antioxidant afforded a similar order of protection to β-carotene as did BHA, whereas little protection was afforded by ascorbic acid.

EXAMPLE 8: Stabilization of Ascorbic Acid

It is known that ascorbic acid is subject to oxidative deterioration, and in particular that its aqueous solutions are rapidly oxidized by air. For the purpose of this example, ascorbic acid was dissolved in 50% ethanol, and the solution, in a closed bottle, was placed in an oven at 60°C for 24 hours. The experiment was repeated with the addition of 0.035 mg or 0.07 mg sodium rosmarinate. Each sample was diluted 1:10 and the ascorbic acid content was analyzed by HPLC on a 250 cm x 4 mm RP-18 column, and eluting with 30:70 acetonitrile/water at a flow rate of 1 ml/min. The peak area absorption at 245 nm, compared with the initial

area, corresponded with the amount of ascorbic acid in each case, the results being as follows:

Sample (10 mg/ml ascorbic acid)	HPLC peak area (245 nm)	% ascorbic acid
control	12,334,252	100
after 60°C, 24 hours (no additive)	5,572,750	45
after 60°C, 24 hours (+0.035 mg rosmarinic acid*)	8,384,409	68
after 60°C, 24 hours (+0.07 mg rosmarinic acid*)	11,130,666	90

*Na salt

These results show that rosmarinic acid (and its derivatives) effectively protects ascorbic acid from decomposition under the given conditions, particularly at the higher concentration of 0.7%. It may be inferred that the extent of this protection would be considerably greater under other conditions, e.g. at ambient temperatures and/or when the ascorbic acid is not necessarily in the form of an aqueous solution.

EXAMPLE 9: Inhibition of oxidation of essential oils

(a) orange essential oil

Different concentrations of romarinate solution were added to 5 ml orange essential oil. The samples were kept at 30°C for seven days, and were then checked for peroxide using the TBA test. The rosmarinate solution ("Organox os") contained 17% propylene glycol, 65% lecithin and 17% sodium rosmarinate (50% pure). Additionally, oxidation at 4°C without antioxidant was noted for comparison. The results were as follows:

Essential oil (TBA)	% Organox os	% rosmarinate	oxidation
5 ml, 30°C	0	0	0.525nm
5 ml, 30°C	0.1	0.008	0.445nm
5 ml, 30°C	0.2	0.017	0.370nm
5 ml, 30°C	0.25	0.021	0.340nm
5 ml, 30°C	0.3	0.025	0.310nm
5 ml, 4°C	0	0	0.320nm

(b) grapefruit oil

This test was carried out similarly to part (a), above, except that the samples were kept for eleven days. The results were as follows:

Essential oil (TBA)	% Organox os	% rosmarinate	oxidation
5 ml, 30°C	0	0	0.150nm
5 ml, 30°C	0.1	0.008	0.105nm
5 ml, 30°C	0.2	0.017	0.080nm
5 ml, 4°C	0	0	0.080nm

(c) orange oil (colorless)

This test was carried out similarly to part (a), above, except that the samples were kept for eleven days. The results were as follows:

Essential oil (TBA)	% Organox os	% rosmarinate	oxidation
5 ml, 30°C	0	0	1.8nm
5 ml, 30°C	0.1	0.008	1.3nm
5 ml, 30°C	0.155	0.013	1.0nm
5 ml, 30°C	0.2	0.017	0.9nm
5 ml, 30°C	0.25	0.021	0.65nm
5 ml, 4°C	0	0	0.70nm

Conclusions By using a relatively small amount of antioxidant at 30°C, it is possible to achieve approximately the same level of inhibition of oxidation, in essential oils, for the given period, compared with refrigeration at 4°C.

While the present invention has been particularly described with reference to certain embodiments, it will be apparent to those skilled in the art that many

modifications and variations may be made. The invention is accordingly not to be construed as limited in any way by such embodiments, rather its concept is to be understood according to the spirit and scope of the claims which follow.

CLAIMS

1. A substance liable to oxidative deterioration, which is characterized by the fact that it incorporates as antioxidant for that substance, at least one compound selected from sub-groups (α), (β) and (γ), namely:

(α) rosmarinic acid;

(β) salts of the carboxylic acid function in rosmarinic acid;

(γ) esters and amides of the carboxylic acid function in rosmarinic acid;

provided that said substance excludes a pharmaceutical formulation, cosmetic composition, animal feedstuff, and a food composition adapted for human consumption, in any of which said at least one compound was incorporated as a pharmacologically active ingredient in presence or absence of any other pharmacologically active ingredient; and that

said at least one compound excludes mixtures in a form as extracted from natural sources and comprising any compound in sub-groups (α), (β and (γ), together with any other antioxidant(s).

2. A substance according to claim 1, further characterized by at least one of the following features:

(a) it incorporates additionally an emulsifying, dispersing or suspending agent;

(b) it incorporates said antioxidant in a form selected from completely water-soluble materials containing a major proportion of non-antioxidant diluent, and which are solid at ambient temperatures, and aqueous solutions thereof;

(c) it is in the form of a powder, tablet, capsule, solution, emulsion, concentrate, syrup, suspension, gel or dispersion;

(d) said antioxidant has been subjected to chromatographic enrichment or purification, prior to incorporation in said substance;

(e) said antioxidant comprises at least one of:

(i) a sodium rosmarinate, preferably isolated from tissue of plants of the *Labiatae* family, most preferably by use of an aqueous extractant; and

(ii) a romarinate salt other than the sodium salt, preferably obtained by cation-exchange with said sodium salt;

(f) said antioxidant constitutes about 0.0001 to about 1.0% by weight of said substance.

3. A substance according to either claim 1 or claim 2, which is selected from sub-groups (I), (II) and (III), namely:

(I) pharmaceutical compositions, cosmetic compositions, animal feedstuff compositions and food compositions adapted for human consumption;

(II) an ingredient, liable to oxidative deterioration, adapted to be incorporated into a composition selected from pharmaceutical compositions, cosmetic compositions, animal feedstuff compositions and food compositions adapted for human consumption;

(III) a composition selected from pharmaceutical compositions, cosmetic compositions, animal feedstuff compositions and food compositions adapted for human consumption, which composition comprises an ingredient liable to oxidative deterioration.

4. A food composition according to claim 3, which is selected from sugar-based confectionery, manufactured cereals, fruit or vegetable products, beverages or beverage concentrates, ground meat products and vegetable analogues thereof.

5. A food composition according to claim 4, which comprises at least one additional ingredient selected from the water-soluble vitamins thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid, cobalamin and ascorbic acid; the oil-soluble vitamins retinol, calciferol, tocopherol and menadione, in combined form the elements sodium, potassium, calcium, phosphorus, magnesium, chlorine, sulfur, iron, copper, iodine, manganese, cobalt, zinc molybdenum, fluorine, selenium and chromium, unsaturated fatty acids which are known to metabolized in the body to prostaglandins, and physiologically compatible derivatives of said fatty acids, food colorants and pigments, acceptable dispersing and suspending agents, and water.

6. A substance according to claim 1 which comprises at least one essential oil.

7. Use of at least one compound selected from sub-groups (α), (β) and (γ), namely:

(α) rosmarinic acid;

(β) salts of the carboxylic acid function in rosmarinic acid;

(γ) esters and amides of the carboxylic acid function in rosmarinic acid;

in admixture with a substance liable to oxidative deterioration, in at least a minimum amount necessary to confer antioxidative protection on said substance, provided that when said substance is a dermato-cosmetic composition, said at least one compound is used in an amount less than 0.1% of the admixture; and that said at least one compound excludes mixtures in a form as extracted from natural sources and comprising any compound in sub-groups (α), (β and (γ), together with any other antioxidant(s).

8. Use according to claim 7, further characterized by at least one of the following features:

(a) said admixture incorporates additionally an emulsifying, dispersing or suspending agent;

(b) said admixture incorporates said antioxidant in a form selected from completely water-soluble materials containing a major proportion of non-antioxidant diluent, and which are solid at ambient temperatures, and aqueous solutions thereof;

(c) said admixture is in the form of a powder, tablet, capsule, solution, emulsion, concentrate, syrup, suspension, gel or dispersion;

(d) said antioxidant has been subjected to chromatographic enrichment or purification, prior to admixture with said substance;

(e) said antioxidant comprises at least one of:

(i) a sodium rosmarinate, preferably isolated from tissue of plants of the *Labiatae* family, most preferably by use of an aqueous extractant, and

(ii) a rosmarinate salt other than the sodium salt, preferably obtained by cation-exchange with said sodium salt;

(f) said antioxidant constitutes about 0.0001 to about 1.0% by weight of said substance.

9. Use according to either claim 7 or claim 8, wherein said substance on which antioxidative protection is conferred is selected from sub-groups (I), (II) and (III), namely:

(I) pharmaceutical compositions, cosmetic compositions, animal feedstuff compositions and food compositions adapted for human consumption;

(II) an ingredient, liable to oxidative deterioration, adapted to be incorporated into a composition selected from pharmaceutical compositions, cosmetic compositions, animal feedstuff compositions and food compositions adapted for human consumption;

(III) a composition selected from pharmaceutical compositions, cosmetic compositions, animal feedstuff compositions and food compositions adapted for human consumption, which composition comprises an ingredient liable to oxidative deterioration.

10. Use according to claim 9, wherein said food composition is selected from sugar-based confectionery, manufactured cereals, fruit or vegetable products, beverages or beverage concentrates, ground meat products and vegetable analogues thereof.

11. Use according to claim 9, wherein said food composition comprises at least one additional ingredient selected from the water-soluble vitamins thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid, cobalamin and ascorbic acid; the oil-soluble vitamins retinol, calciferol, tocopherol and menadione, in combined form the elements sodium, potassium, calcium, phosphorus, magnesium, chlorine, sulfur, iron, copper, iodine, manganese, cobalt, zinc molybdenum, fluorine, selenium and chromium, unsaturated fatty acids which are known to metabolized in the body to prostaglandins, and physiologically compatible derivatives of said fatty acids, food colorants and pigments, acceptable dispersing and suspending agents, and water.

12. Use according to claim 7, wherein said substance comprises at least one essential oil.

13. Method for treating a substance liable to oxidative deterioration, in order to confer antioxidative protection on said substance, which comprises admixing said substance with at least one compound selected from sub-groups (α), (β) and (γ), namely:

(α) rosmarinic acid;

(β) salts of the carboxylic acid function in rosmarinic acid;

(γ) esters and amides of the carboxylic acid function in rosmarinic acid;

in at least a minimum amount necessary to confer said protection on said substance, provided that when said substance is a dermato-cosmetic composition, said at least one compound is used in an amount less than 0.1% of the admixture; and that said at least one compound excludes mixtures in a form as extracted from natural sources and comprising any compound in sub-groups (α), (β) and (γ), together with any other antioxidant(s).

14. Method according to claim 13, further characterized by at least one of the following features:

(a) said admixture incorporates additionally an emulsifying, dispersing or suspending agent;

(b) said admixture incorporates said antioxidant in a form selected from completely water-soluble materials containing a major proportion of non-antioxidant diluent, and which are solid at ambient temperatures, and aqueous solutions thereof;

(c) said admixture is in the form of a powder, tablet, capsule, solution, emulsion, concentrate, syrup, suspension, gel or dispersion;

(d) said antioxidant has been subjected to chromatographic enrichment or purification, prior to admixture with said substance;

(e) said antioxidant comprises at least one of:

(i) a sodium rosmarinate, preferably isolated from tissue of plants of the *Labiatae* family, most preferably by use of an aqueous extractant, and

(ii) a rosmarinate salt other than the sodium salt, preferably obtained by cation-exchange with said sodium salt;

(f) said antioxidant constitutes about 0.0001 to about 1.0% by weight of said substance.

15. Method according to either claim 13 or claim 14, wherein said substance on which antioxidative protection is conferred is selected from sub-groups (I), (II) and (III), namely:

(I) pharmaceutical compositions, cosmetic compositions, animal feedstuff compositions and food compositions adapted for human consumption;

(II) an ingredient, liable to oxidative deterioration, adapted to be incorporated into a composition selected from pharmaceutical compositions, cosmetic compositions, animal feedstuff compositions and food compositions adapted for human consumption;

(III) a composition selected from pharmaceutical compositions, cosmetic compositions, animal feedstuff compositions and food compositions adapted for human consumption, which composition comprises an ingredient liable to oxidative deterioration.

16. Method according to claim 14, wherein said food composition is selected from sugar-based confectionery, manufactured cereals, fruit or vegetable products, beverages or beverage concentrates, ground meat products and vegetable analogues thereof.

17. Method according to claim 15, wherein said food composition comprises at least one additional ingredient selected from the water-soluble vitamins thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid, cobalamin and ascorbic acid; the oil-soluble vitamins retinol, calciferol, tocopherol and menadione, in combined form the elements sodium, potassium, calcium, phosphorus, magnesium, chlorine, sulfur, iron, copper, iodine, manganese, cobalt, zinc molybdenum, fluorine, selenium and chromium, unsaturated fatty acids which are known to metabolized in the body to prostaglandins, and physiologically compatible derivatives of said fatty acids, food colorants and pigments, acceptable dispersing and suspending agents, and water.

18. Method according to claim 13, wherein said substance comprises at least one essential oil.

19. A substance according to claim 3, wherein said ingredient comprises at least one member selected from the group consisting of vitamins, their functional derivatives, and analogs thereof.

20. Use according to claim 9, wherein said ingredient comprises at least one member selected from the group consisting of vitamins, their functional derivatives, and analogs thereof.

21. Method according to claim 15, wherein said ingredient comprises at least one member selected from the group consisting of vitamins, their functional derivatives, and analogs thereof.

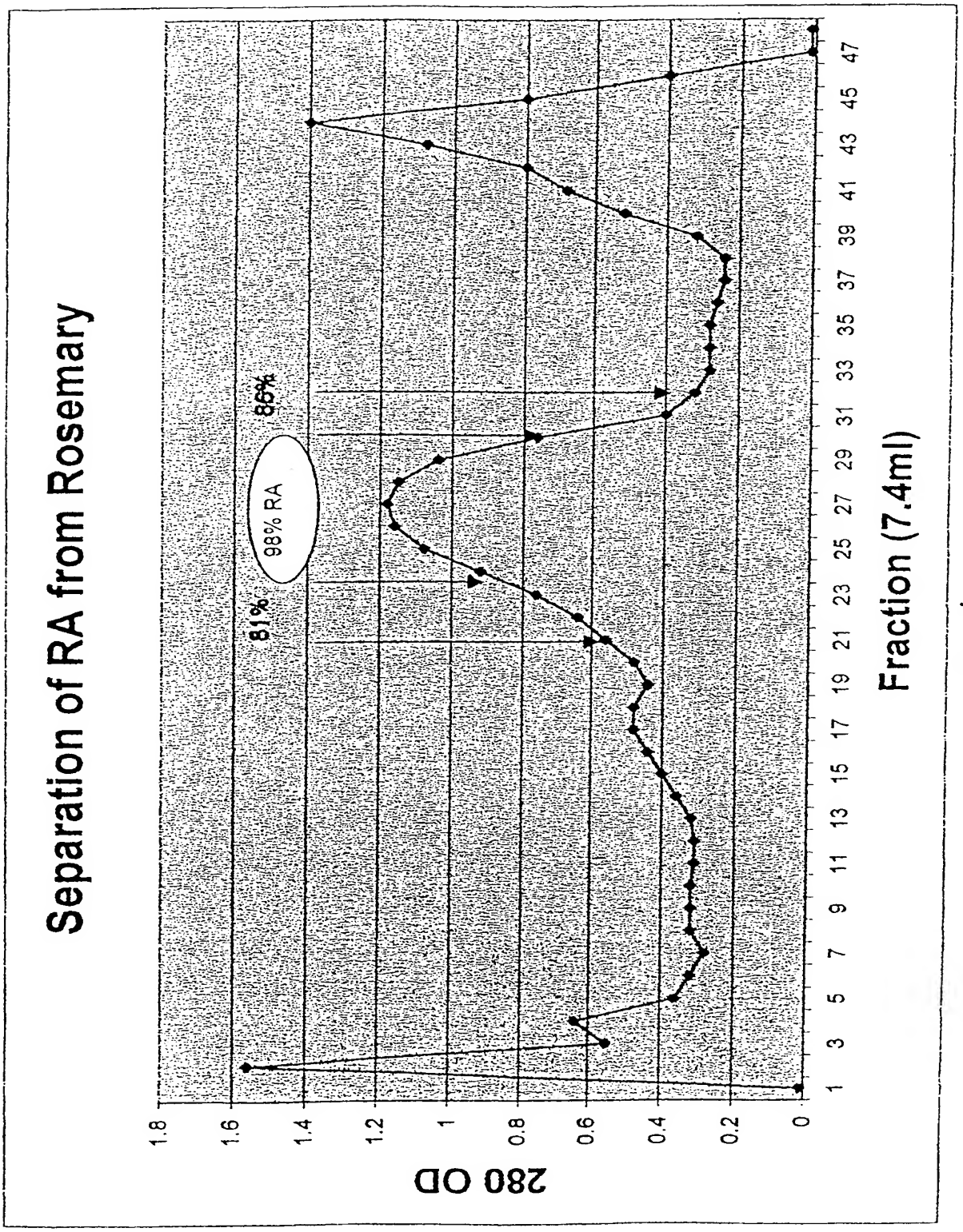

Separation of RA from Rosemary
280 OD
Fraction (7.4ml)
81%
98% RA
86%
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47

Fig. 1

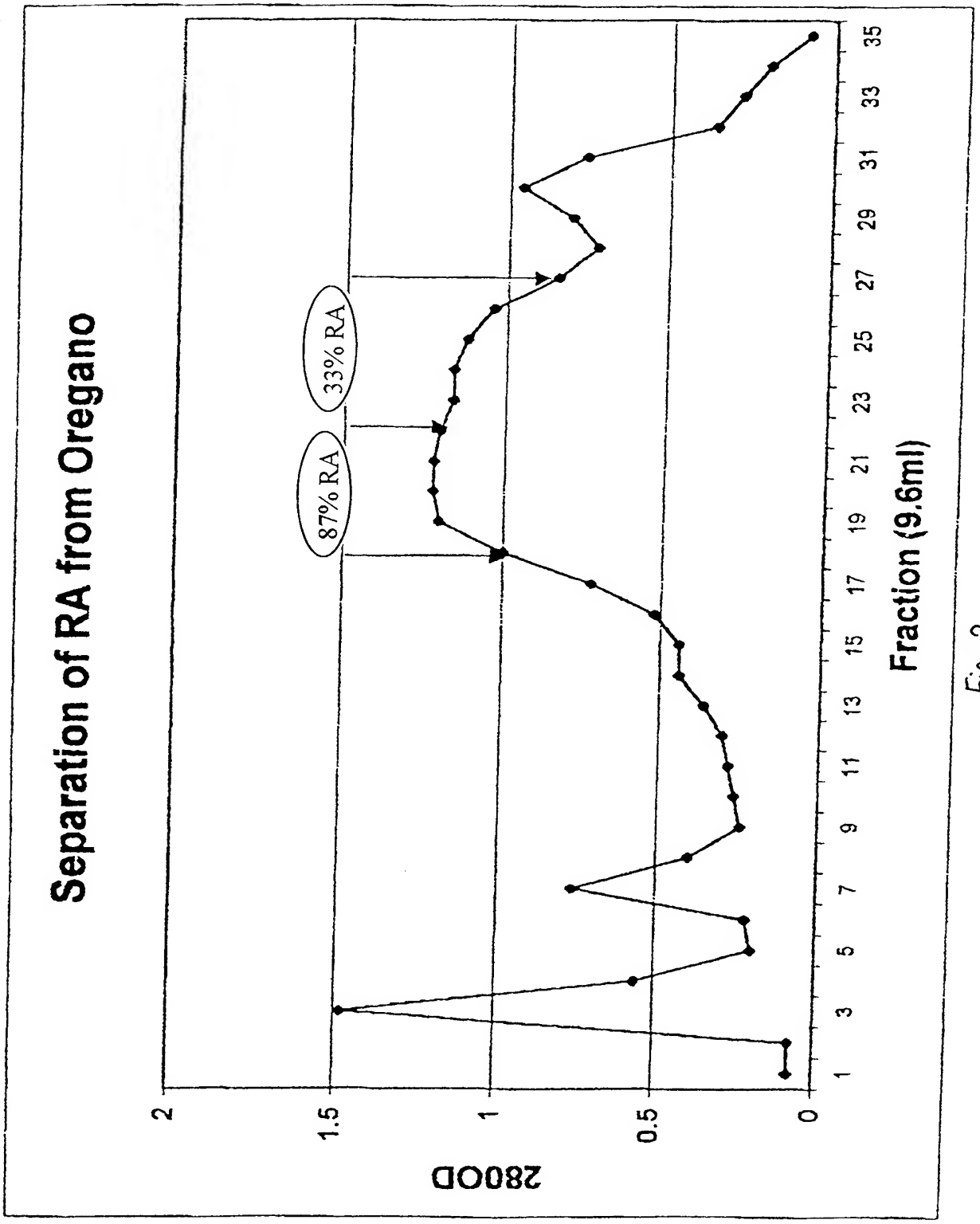

Separation of RA from Oregano
280OD
2
1.5
1
0.5
0
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
Fraction (9.6ml)
87% RA
33% RA

Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 99/00693

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C09K15/08 A23L3/3508 A61K7/48 A23L1/221

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C09K A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEN J H ET AL: "ANTIOXIDANT ACTIVITIES OF CAFFEIC ACID AND ITS RELATED HYDROXYCINNAMIC ACID COMPOUNDS" JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. WASHINGTON, vol. 45, no. 7, 1 July 1997 (1997-07-01), pages 2374-2378, XP000656898 ISSN: 0021-8561 table 1 ---- -/--	1,7,13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 2000	05/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Shade, M

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INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/IL 99/00693

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FRANKEL E N ET AL: "ANTIOXIDANT ACTIVITY OF A ROSEMARY EXTRACT AND ITS CONSTITUENTS, CARNOSIC ACID, CARNOSOL, AND ROSMARINIC ACID, IN BULK OIL AND OIL-IN-WATER EMULSION" JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. WASHINGTON, vol. 44, no. 1, 1 January 1996 (1996-01-01), pages 131-135, XP000546463 ISSN: 0021-8561 table 1	1,7,13
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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